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Position statement of the ZKBS on the present state of scientific knowledge concerning the effect of Bt toxins in mammals

At present, the genetically modified crops cultivated worldwide are mainly those that are characterised by tolerance to herbicides or by expressing proteins referred to as Bt toxins that reduce the plants' sensitivity to pests. In Germany, the Bt maize MON810 had been used in agriculture as a genetically modified plant until the ban on cultivation in 2009. In view of the publication of controversial results of various animal feeding studies, the ZKBS summarises in this position statement the present state of scientific knowledge concerning the mechanism of action of Bt toxins and the possible effects in mammals, in particular the effect on microorganisms and the gastrointestinal mucosa.

Bt-Toxin

Bacillus thuringiensis (*Bt*) is a gram-positive soil bacterium belonging to the family of *Bacillaceae* that is allocated to risk group 1 as a donor and recipient organism for genetic engineering operations in accordance with Sec. 5 (6) in conjunction with Appendix I of the Genetic Engineering Safety Regulations (GenTSV). During sporulation, the bacteria produce crystal-like, parasporal inclusions containing proteins that belong to the δ-endotoxins. These are encoded by what are called *cry* (*crystalline*) or *cyt* (*cytolytic*) genes. The various δ-endotoxins are toxic to a wide range of insects and nematodes, but not to mammals. There are collectively referred to herein as Cry proteins or Bt toxins (activated Cry proteins). Due to their specificity of action, formulations of sporulating bacteria have already been used in agriculture as herbicides since the first half of the 20th century (Glare & O´Callaghan, 2000). The Cry proteins distinguish themselves from many chemical insecticides by their rapid biological degradation (Entwistle *et al*., 1993; de Maagd *et al*., 2003). In addition, the Cry proteins produced by different *Bt* strains each have a narrow range of action that is mostly limited to individual orders, families or species groups (van Frankenhuyzen, 2009). By developing genetically modified plants that produce Bt toxins, the positive characteristics of Bt toxins were combined with improvements in plant protection management (improved resistance to pests, easy application, no additional effort) (Shelton *et al*., 2002; James 2009).

The Cry proteins that are encoded on plasmids in Bt strains are of special interest for plant protection. To date, at least 335 different variants of these proteins with a specific toxic effect on certain groups of insects have been described, according to which they were classified into various subfamilies by Höfte & Whiteley (1989). Cry1 proteins show an effect against Lepidoptera (butterflies), Cry2 proteins against Lepidoptera and Diptera (butterflies and flies), Cry3 proteins against Coleoptera (beetles) and Cry4 proteins against Diptera (flies). Later on, Crickmore *et al.* (1998) introduced a new nomenclature based on similarities between the amino acid sequences of the Cry proteins.

The Cry proteins are present in the sporulating bacterial cell as crystalline protoxins in inclusions. After ingestion by the insects, the protoxin is dissolved in the intestines of the insects (pH shift) and proteolytically activated. Proteolytic activation takes place through cleavage of the C-terminal region and results in a toxic protein. The toxin so activated then binds to specific

receptors that are located on the apical side of the microvilli of the epithelial cells in the midgut of insects. For the subsequent step, the insertion of the toxin into the cell membrane involving the formation of membrane pores, various alternative mechanisms are being discussed. On the one hand, the binding of monomers to the receptors is regarded as sufficient to cause the formation of pores in the cell membrane. On the other, a local accumulation of the activated toxin and/or the oligomerisation of the monomers are regarded as prerequisites for the initiation of pore formation (Pigott & Ellar, 2007; Bravo *et al*., 2004). As a result of pore formation (Gazit *et al*., 1998; Soberon *et al*., 2010), the permeability to inorganic ions, amino acids and sugar increases, ultimately resulting in osmolysis of the epithelial cells. The intestinal function is thereby destroyed and the insect dies. In addition to this mechanism, a signal transduction following receptor activation through the binding of the toxin, which results in cell death (oncosis) and also leads to destruction of the intestinal function, has been proposed (Zhang *et al*., 2006; Vachon *et al*., 2012).

There are few similarities between the amino acid sequences of the individual Cry protein families. However, x-ray crystallography has revealed homologous structures within the Cry protein families. So far, the structure of seven activated Cry proteins of various classes has been solved (Li *et al*., 1991; Grochulski *et al*., 1995; Galitsky *et al*., 2001; Morse *et al*., 2001; Boonserm *et al*., 2005, 2006). According to this, the proteins of approx. 60 kDa in size consist of three domains. Domain I consisting of an alpha-helical bundle is assumed to play a role in membrane insertion and pore formation (Schnepf *et al*., 1998). Domain II and III are two different β-sheet structures that bind to the respective receptor(s) (Pigott & Ellar, 2007).

The host specificity of a Cry protein firstly depends on the pH-dependent solubilisation and the proteolytic activation actually taking place in the intestines of the respective insect. The second prerequisite for the toxic effect is the ability of the Bt toxin to bind to specific receptors.

In the development of genetically modified crops, Cry proteins that are specific to butterflies (Lepidoptera) and beetles (Coleoptera) are used *(http://ec.europa.eu/food/dyna/gm_register/index_en.cfm)*. One or more nucleic acid segments that code for the already activated forms of the Cry proteins are inserted into the plant genome. These may also be composite proteins consisting of different domains of different Bt toxins (synthetic Bt toxins). The expression is under the control of the specific plant so that the toxin can be expressed by the genetically modified plant and is present in the cytosol in dissolved form. The pH-dependent solubilisation and proteolytic digestion are no longer required for the functionality of the produced toxin. Binding to a specific, functional receptor is the decisive factor for the toxic effect.

The specificity of interaction between Bt toxin and receptor has been demonstrated using various methods (site directed mutagenesis, surface plasmon resonance spectroscopy, competitive binding studies using peptides or antibody fragments including Biacore studies and interaction studies between protein fragments). Cry proteins have been observed to interact with various membrane-bound components, such as glycosylphosphatidylinositol (GPI)-anchored aminopeptidase N (APN), alkaline phosphatases and glycolipids. However, only interactions with cadherin-like membrane proteins led to a toxic effect of the Bt toxins (Nagamatsu *et al*., 1999). These are receptor structures that are only found in this conformation in the intestinal cell membrane of insects (Lecuit *et al*., 2007; Midboe *et al*., 2003). It was only the ectopic expression of the receptor proteins of insects in mammalian cells that made them sensitive to the Bt toxin (Tsuda *et al*., 2003).

Possible effect of the Bt toxin on microorganisms

A toxic effect on prokaryotes comparable to the toxic effect on insects is not expected. Prokaryotic cells differ from eukaryotic cells, in particular from animal cells, in terms of structure. In

contrast to animal cells, the bacterial cytoplasm membrane with the proteins integrated therein is surrounded by a complex cell wall. This consists of various layers of the peptidoglycan murein and is permeable to low-molecular-weight substances and salts. Gram-negative bacteria are additionally surrounded by another outer membrane. It also contains membrane proteins that serve predominantly to transport substances. There is no evidence to suggest the presence of cadherin-like proteins with a binding site for Bt toxins in the cytoplasm or outer membrane of bacteria.

In-vitro and *in-situ* studies demonstrate that the Bt toxin expressed by the genetically modified plant can enter the surrounding soil via the roots of the plant, where the water-soluble protein can bind to surface-active particles in the soil (Saxena & Stotzky 2000) and, in contrast to soil water, remain detectable over longer periods of time (up to 180 days) (Saxena and Stotzky 2001a). The diversity and activity of bacterial communities in the soil is the highest in the plant rhizosphere. To investigate a possible effect of the Bt toxin on bacterial communities, the above-mentioned areas were examined in studies. With the help of taxon-specific quantitative PCR (Fierer *et al*., 2005), fingerprinting and pyrosequencing (Dohrmann et al., 2013), the quantity and diversity of soil bacteria was examined in various geographical regions where various genetically modified plants carrying the *cry* gene were cultivated. There was no evidence to suggest an effect of the Bt toxin (Singh *et al*., 2013; Barriuso *et al.,* 2012; Devare *et al*., 2007; Baumgarte & Tebbe, 2005, Miethling-Graff *et al*., 2010; Dohrmann *et al*., 2013, Oliveira *et al*., 2008). Although minor differences in the composition of the bacterial communities were found between genetically modified and control plants in isolated cases, these can also be attributed to other factors such as weather conditions and the stage of growth of the individual plant, in particular the respective development of the rhizosphere. In addition, the metabolites released by the respective plant via the rhizosphere also have an influence on the bacterial communities. For example, the bacterial diversity differs not only in the rhizosphere of individual plants (Buee *et al*., 2009) but also in various areas of the rhizosphere of a single plant (Watt *et al*., 2006).

Possible effect of the Bt toxin on the gastrointestinal tract of mammals

Compared to mammals, the digestive system of insects has a simple structure and is subdivided into three functional sections referred to as foregut, midgut and hindgut. The midgut is the place of activation and binding of the Bt toxin and the associated toxic effect. It is lined with glandular epithelium that produces enzymes required for digestion. The midgut is covered with microvilli featuring cadherin-like receptors and has a neutral to alkaline pH value.

The digestive system of mammals substantially differs from that of insects. In mammals, the ingested food passes down the oesophagus into the stomach. In mammals with a simple gastrointestinal tract, protein constituents in the food are denatured by pepsin and hydrochloric acid (pH<2) and proteolytically degraded in the stomach before they are transported to the intestinal tract. Ruminants are characterised by additional upstream chambers of the stomach, in particular the rumen (paunch). In these chambers, the vegetable food is pre-fermented with the help of microorganisms. Bt toxins, including synthetic Bt toxins, also undergo these processes. The degradation of Bt toxins contained in maize in the gastrointestinal tract of both ruminants and omnivores has been demonstrated in a number of studies (Paul *et al*., 2010; Chowdhury *et al*., 2003; Einspanier *et al*., 2004; Lutz *et al*., 2005; EFSA-Report, 2008; Walsh *et al*., 2012b; EFSA-Opinion, 2008).

- Effects on the mucosa

Although the Bt toxins ingested with food are denatured and degraded in the stomach of mammals, *in-vitro* experiments have been performed in cell culture systems to examine the possible effects of Bt toxins on the cells of the intestinal mucosa. The predominant epithelial cells in the intestinal mucosa (enterocytes or intestinal absorptive cells) are polarised and covered with microvilli to increase the surface area. Besides the epithelial cells, the tissue surface also contains other cells, such as immune or endocrine cells. They are additionally surrounded by a glycocalyx (sugar coat) to protect them against self-digestion. The number of studies in which the effects of Bt toxins on functional intestinal mucosa were tested *in vitro* is small, since such a cell culture system is very difficult to establish (Cencič & Langerholc, 2010). Shimada *et al.* (2006) generated membrane vesicles from the epithelial cells of the small intestines of cows and pigs and tested the binding affinity of Bt toxins to the respective cell membrane as well as their potential toxic effect. Even in cases where a small proportion of the Bt toxins bound to the membranes independently of a cadherin-like receptor, a change in the membrane and an associated toxic effect were not observed. Vazquez-Pedron *et al.* (2000) detected six different Cry1Ac-binding proteins in small intestinal specimens of mice using immunological methods. However, these do not function as receptors. Although the interaction with the Cry proteins induced a temporary increase in membrane stress, an increase in the permeability of the membrane, which is associated with tissue destruction, was not observed. Bondzio *et al*. (2013) used a more recent technology referred to as real-time cell analysis to analyse the response of intestinal cells of pigs to exposure to the Bt toxin over a longer period of time *in vitro*. Despite high concentrations of Bt toxins, a reduced viability of the cells was not observed.

In the rumen of cows, both fragmented and complete Bt toxins can still be detected a few hours after food ingestion (Wiedemann *et al*., 2006). However, the introduction of Bt toxins in physiological concentrations in *in-vitro* experiments with cultures of isolated rumen epithelial cells of sheep did not show an effect on cell viability (Bondzio *et al.,* 2008). The introduction of unphysiologically high concentrations was observed to cause spontaneous insertion of Bt toxins into the membrane of the epithelial cells. However, this did not have an influence on cell viability (Stumpff *et al.,* 2007).

- Effects on microbiomes

Mammals are colonised by a great number of microorganisms, mainly consisting of bacteria (microbiomes). Besides the skin and the nasopharynx, the gastrointestinal tract is among the areas that are most densely colonised by microorganisms. The diversity of bacteria in the intestines attracts increasing attention, in particular as regards their relevance for human health. In late 2007, the U.S. National Institute of Health (NIH) established the Human Microbiome Project (HMP) to sequence the genomes of all microorganisms that colonise humans. Within five years, a total of 5,177 taxonomical bacterial profiles of 242 subjects from mainly western societies were generated by the consortium consisting of 80 research groups (HMP-Consortium 2012 a, b). In the course of this project, it has become apparent that the composition of a microbiome is influenced by a number of parameters, such as pH value, humidity, oxygen content, host factors, such as genetic disposition, immunology, nutritional state, age and ethnic origin, as well as interactions among bacteria. Microbiomes vary greatly between, and even within, species. In this connection, pigs are suitable models because of their physiological similarity to humans as regards the digestive system and associated processes. They are also omnivores and are comparable to humans in terms of size/weight and nutritional requirements. Their intestinal microbial ecosystem also resembles that of humans (Heinritz *et al*., 2013). To that extent, studies with pigs can also be drawn on to predict the effects in humans.

Bt toxins ingested with food are not assumed to have a toxic effect on the microbiome of omnivores and ruminants. As described above, the gastrointestinal tract of omnivores is designed in such as way that the food is largely denatured and proteolytically degraded in the acidic environment of the stomach before the digested food reaches the intestines. Therefore, extended contact between the intact Bt toxin and the microbiome is not expected to occur. In ruminants, the food containing Bt toxins already comes in contact with microbial communities in the rumen, i.e. before denaturation in the acidic environment. A toxic effect on the microbiome is nevertheless not assumed, given that the Bt toxin-specific receptor structures found in the epithelial cells of insects were not detected in bacterial membranes (see above).

These assumptions have been substantiated in a number of feeding studies with both omnivores and ruminants. The ingestion of food containing Bt toxins was not observed to have a significant influence on the bacterial communities in the intestines in any of the studies (Einspanier *et al.*, 2004; Wiedemann *et al.*, 2007; Trabalza-Marinucci *et al.*, 2008; Buzoianu *et al.*, 2012a; Buzoianu *et al.*, 2012b; Yuan *et al.*, 2013).

Conclusion

In the ZKBS' opinion, there is extensive, sound knowledge of the effect of Cry proteins on various animal groups. Although very specific details of the mechanism of action of Bt toxins are currently being discussed and examined in greater detail, the principle of the mechanism of action itself and the host specificity are not in question. Based on the knowledge of the mechanism of action and the absence of Bt toxin receptors in the intestines of mammals, Bt toxins are not expected to have an effect on mammals. This is additionally substantiated by numerous feeding studies. A great number of studies investigating the effect of Bt toxins on bacterial communities in the digestive tract of mammals did not demonstrate an effect on their composition.

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